

Controlling membrane permeability with bacterial porins: application to encapsulated enzymes

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Abstract

Recent achievements of membrane protein science allow easy protein modification by genetic engineering and, for some proteins, their production in large quantities. We regard these features as the basic requirements for applications of membrane proteins in materials science. Here, we demonstrate a possible application of membrane proteins, inserting porins from the outer cell wall of *Escherichia coli* into the walls of liposomes. Encapsulation of enzymes into liposomes or polymer nanocapsules protects them against proteases and denaturation. Functional reconstitution of porins into the capsule shell allows to control the rate and selectivity of substrate permeation, and thus to control the enzyme reaction kinetics. We suggest that this technique can prove to be useful in the area of biosensors, providing enzymatic stability while keeping the functionality or even enhancing the sensitivity by substrate preselection. Another application of this kind of stabilisation is in the field of single enzyme activity recording. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

One of the important research goals in current materials science is to devise new types of respon-

sive materials on a nanometer scale [1]. Although in the laboratory quite a challenge, nature already provides optimised solutions for this problem since the origin of life. For example, the survival of bacteria requires a continuous exchange of material across the cell wall [2]. Gram-negative bacteria possess two separate cell walls. The inner one contains a dense lipid bilayer, which is electrically tight, so that no ions or other hydrophilic substrates can cross the barrier without the help

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of highly specific membrane proteins. This inner cell wall is separated from the outer one by an aqueous phase filled with water-soluble polymers, the peptidoglycan. In contrast, the outer cell wall is fairly permeable to smaller solutes below a molecular weight of about 400 Da. Such substances can freely permeate under a concentration gradient through general diffusion porins in the outer cell wall. The most prominent of the general diffusion porins is OmpF (Outer membrane protein F) [3,4]. However, under stress, for example in case of lack of nutrition, the pure diffusion process is too slow and the bacteria need to enhance the efficiency of the translocation. For those cases, nature has created a series of rather specific and highly sophisticated membrane channels. The most extensively studied example is the malto-oligosaccharide specific channel LamB, or Maltoporin, of *Escherichia coli*. Furthermore, nature has also designed porins, which open and close upon a specific trigger, or requiring ATP.

Porins are attractive candidates to create new types of materials because they are very stable. For example, OmpF is not denatured in 4 M GuaHCL, 70° C or in 2% SDS. Moreover, recombinant technology permits their production in *E. coli* with high yields (tens of mg per liter of culture). A third advantage is the availability of the high-resolution 3-D crystal structure showing details of substrate binding sites, which facilitates enormously the engineering of modified proteins [3]. A fourth advantage is the accessibility of thermodynamic and kinetic information on substrate translocation across the channel. Reconstituted porins in lipid or block copolymer membranes allow observation of the crossing of a single substrate molecule through the porin channel. This approach is a further development of a technique based on high-resolution measurements of ion currents through a single channel to probe neutral solute transport [5]. For example, in our recent studies [6–8], we used Maltoporin, a specific membrane channel that facilitates diffusion of malto-oligosaccharides across the outer bacterial cell wall. This type of measurement can be generalised to screen for facilitated transport of a wide variety of molecules [9], e.g. to study antibiotic uptake through porins, to test for toxins

present in the aqueous environment, to create sensors for DNA sequencing, or to detect analytes in solution [10], just to name a few.

We describe an application of porin reconstitution into enzyme-containing vesicles to control their permeability of the capsule wall and thus the enzymatic activity. In Fig. 1, we schematically show such a capsule. We have used the general diffusion porin OmpF as a model system, which allows the permeation of small molecules with molecular weights below 400 Da. The modification of the permeability was revealed by probing the enzymatic activity [11]. The first encapsulated enzyme was beta-lactamase responsible for beta-lactam antibiotic resistance, and the second one was based on acetylcholinesterase, which is the target of many insecticides [12–17]. Obviously, the latter can be applied to construct a rapid screening test for specific insecticides.

Liposomes can be easily prepared using the extrusion technique. However, such liposomes are generally rather unstable [1]. Stabilisation of the liposomes by polymerisation of reactive lipid molecules is feasible, but it destroys the lateral mobility of the lipids required for the functionality of certain membrane proteins. Recently, it was shown that polyelectrolyte multilayer nanocapsules could be coated with lipid membranes [18]. Such supported vesicles are more stable, but due to interactions with the underlying polyelectrolyte skeleton, their shells have frequent defects, i.e. they are not completely tight. Here in this work, we suggest different types of stabilization. One possibility is to devise block copolymer vesicles,

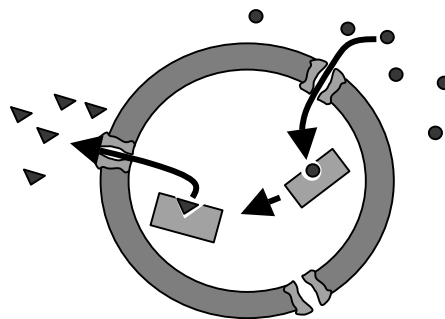


Fig. 1. Schematic representation of a polymer-stabilised nanoreactor with the encapsulated enzyme.

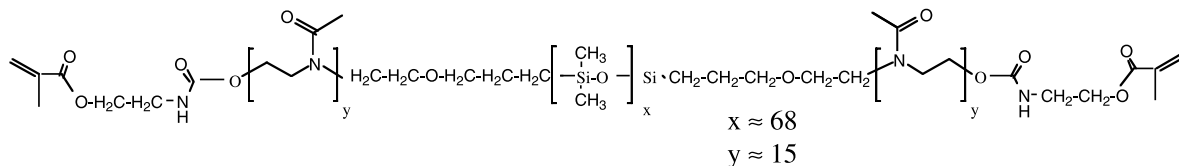


Fig. 2. Structure of a new type of an amphiphilic ABA triblock copolymer. The polymer consists of a flexible, hydrophobic poly-(dimethylsiloxane) (PDMS) middle block and two water soluble poly-(2-methyloxazoline) (PMOXA) side blocks. Additionally, the ends of this PMOXA–PDMS–PMOXA triblock copolymer carry methacrylate groups, which allow a crosslinking polymerisation.

which later can be cross-linked via polymerizable groups at the water-soluble blocks [11], or to add hydrophobic methacrylate monomers to aqueous liposome dispersions, which penetrate into the hydrophobic interior of the lipid membranes [19,20]. These monomers can easily be polymerized inside the membranes by UV-induced free radical polymerization. Interestingly, both methods allow preparation of stable polymer nanocapsules, in which encapsulated or inserted proteins preserve their full activity.

2. How to incorporate porin in a capsule shell

Liposomes were formed with palmitoyl-oleoyl-phosphocholine (POPC) purchased from Avanti Polar Lipids Inc. (Birmingham, AL, USA) or with 'synthetic lipids' called ABA triblock copolymers. The latter are comprised of three parts, a hydrophobic poly(dimethylsiloxane) block located at the center of the molecule and two hydrophilic poly(2-methyloxazolin) blocks located at each extremity (for details see Fig. 2). The polymer additionally carries reactive methacrylate end groups, which can be polymerised using UV-induced free-radical polymerisation. In aqueous solution, these molecules spontaneously associate to form two-dimensional layers, which can be arranged in liposome-like nanocapsules [11]. It was shown that the subsequent UV-induced polymerisation of the reactive polymer does not alter the morphology of the underlying aggregates.

The protein, OmpF from the outer membrane of *E. coli*, was purified according to the previous protocol [4] and stored at 1 mg ml⁻¹ in 1% octyl-POE detergent (Alexis, Lauchringen,

Switzerland). About 10 mg of POPC or ABA triblock solution in chloroform was evaporated under high vacuum during 6 h. After drying, 20 µl of the OmpF stock solution was added, vortexed and dried again for a short period (Molar ratio POPC:OmpF 10 000:1 × 10⁴). The dry mixed POPC, ABA triblock/protein film was dispersed in 1 ml buffer containing 10 mM Hepes, 100 mM NaCl. This yielded a dispersion containing multilamellar, polydisperse liposomes. The dispersion was treated by a freeze-thaw cycle consisting of five times freezing in liquid nitrogen and thawing in a water bath at 30° C. To obtain unilamellar liposomes of rather uniform size, the dispersion was repeatedly extruded through polycarbonate filters (Nucleopore filter, Millipore) with a pore size of 100 nm. This led to 120 nm diameter liposomes containing 10 porins on the average.

3. Polymerisation of hydrophobic monomers inside the lipid membranes

About 5 ml (2 mg ml⁻¹ with respect to the lipid) of the liposome solution was mixed with 1.01 mg (9 × 10⁻⁴ mol) of *n*-butyl methacrylate (BMA, Fluka) and 0.7 mg (4.5 × 10⁻⁴ mol) of the cross-linking agent ethylene glycole dimethacrylate (EGDMA, Fluka). Purified argon was bubbled through the solution to eliminate oxygen. The polymerisation was initiated by UV-irradiation (Ultratech 400 W, λ = 254 nm, Osram AG) for 30 min. Previous investigations showed a nearly complete conversion of the monomers under these conditions [20]. Moreover, both the inserted porins and the encapsulated enzymes preserved their activity during the polymerisation.

4. How to characterise the passage of molecules through porins

The most appropriate method to characterise channel-forming membrane proteins is conductance measurements [2,5–9]. The measurement cell consists of two chambers separated by a hole (less than 0.1 mm diameter) in a thin poly(tetrafluorethylene) film sandwiched between two half-cells made of Teflon. Prior to each measurement, this hole has to be pretreated to render it lipophilic by coating it with a hexadecane/hexane (0.5% v:v) droplet. After allowing for hexane evaporation, each chamber is filled with 1.5 ml buffer (1 M KCl, 1 mM CaCl_2 , 10 mM Tris, pH 7.4). Black lipid bilayers or black polymer membranes were formed according to the classical Montal-Mueller technique by spreading lipids in hexane/chloroform (9:1) or triblock copolymers in toluene/chloroform (1:1) across the aqueous buffer. In the case of lipids, we used diphytanoylphosphatidylcholine (DphPC, Avanti Polar Lipids) for stability reasons. After 20 min of evaporation, the buffer level is lowered below the hole level and raised again. Typically, after the first or second trial, a stable unilamellar membrane is formed.

In order to insert single porin trimers in reasonable amounts of time, yet avoiding multiple insertions, a careful balance between the concentration of the protein solution, detergent concentration and buffer volume has to be found. One single porin trimer has to find the membrane and to insert, while all the others must be inactivated, e.g. by precipitation. Maltoporin from the stock (1 mg ml^{-1} in 1% octyl-polyoxyethylene (OPOE), Alexis, Lauchringen, Switzerland) was diluted 10^2 – 10^5 times in the buffer containing 1% OPOE. Insertion was optimal if smallest amounts (less than 1 μl) were injected into the *cis* side of the cell held at a positive potential.

Membrane current was measured via homemade Ag/AgCl electrodes. One electrode was used as ground and the other connected to the headstage of an Axopatch 200B amplifier (Axon Instruments, USA), allowing the application of adjustable potentials (typically, 100 mV) across the membrane. In the absence of substrate, the

conductance fluctuates around 200 pS at 1 M KCl. In Fig. 3a, we show the effect of small amounts of substrate (about 1.8×10^{-6} M Maltotetraose). A few isolated closures of a single monomer and one simultaneous closure of two monomers are observed. Fig. 3b shows the same preparation after addition of a higher concentration (20×10^{-6} M) of Maltotetraose, leading to an avalanche of single and multiple closures of the trimeric channel. An analysis of the ion current fluctuations between the open to the closed state as a function of concentration yields readily the on- and off-rate of the substrate into the channel lumen [6–8]. In the present case of maltotetraose, we obtained $k_{\text{on}} = 11 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and $k_{\text{off}} = 695 \text{ s}^{-1}$, which corresponds to an affinity constant of $K = k_{\text{on}}/k_{\text{off}} = 16\,900 \text{ M}^{-1}$. These results do agree with earlier multichannel recordings [8]. The same measurement can also be performed with polymerized triblock copolymer membranes,

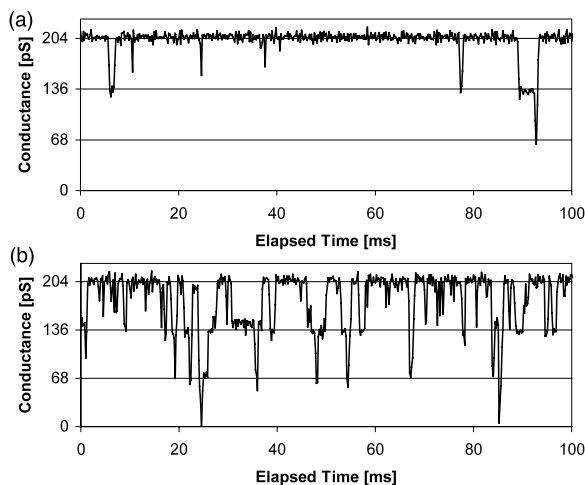


Fig. 3. Ion current recording through a single Maltoporin channel. In the absence of Maltotetraose, we observe a flat line with about 200 pS conductance in 1 M KCl solution. Addition of Maltotetraose in small concentrations of (a) 1.8 μM , and (b) 30 μM to the aqueous buffer causes individual sugar molecules to penetrate the channel and to block it for ions. The higher the concentration, the more often a blockage occurs. At concentrations high enough, simultaneous closures of two monomers of the trimer or even complete closures are found. Analysis of the ion current fluctuations yields the on- and off-rates of the sugar binding to the channel lumen.

Table 1
Apparent enzymatic activity of acetylcholinesterase in different formulations

Substrate concentration	Free enzyme (s^{-1})	Capsule in absence of porin (s^{-1})	Capsule in presence of porin (s^{-1})
10 μM	285	11	183
100 μM	677	70	572
1 mM	919	147	926
10 mM	714	137	753

and the sugar affinity constants are in good agreement with those obtained in conventional lipid membranes [21]. This indicates that the protein remains fully functional in copolymer membranes despite the expected mismatch of their hydrophobic-hydrophilic pattern; the hydrophobic-hydrophilic pattern of Maltoporin is naturally optimized for the much thinner biological membrane. It seems that the high flexibility and the conformational freedom of the polymer molecules allow a block to adapt to the specific geometry and dynamic requirements of the membrane protein. Since the polymerisable groups of the polymer are attached to the very ends of the hydrophilic blocks, the hydrophobic PDMS middle block preserves certain mobility within the membrane even after the crosslinking reaction.

5. Encapsulation of enzymes in liposomes containing porins

Encapsulation of enzymes was performed by adding the protein to the buffer prior to liposome formation. The number of enzyme molecules in each nanocapsule depends on the enzyme concentration during the encapsulation and is proportional to the volume of buffer encapsulated versus the volume of buffer remaining outside the liposomes. Non-encapsulated enzyme molecules were removed chromatographically either by gel filtration on Sephadex G-150 (Pharmacia) or by inverse affinity. In this latter case, solution containing liposomes is loaded on an affinity column. Free enzyme molecules bind to the column ligand while the encapsulated enzyme passes without any reaction on the column and is recovered. A different possibility is to enzymati-

cally digest the accessible enzyme by adding sufficiently high concentration of pronase. We encapsulated the enzyme β -lactamase, which is able to hydrolyse β -lactam antibiotics like ampicillin. The activity of the enzyme can be quantified via a secondary reaction taking advantage of a property of the product of ampicillin hydrolysis, ampicillinoic acid, which can reduce iodine to iodide. This reaction can readily be monitored by iodometry, i.e. via the decolorization of a starch-iodine complex [12,13]. More recently, we also encapsulated acetylcholinesterase (AChE), an enzyme which hydrolyses the neurotransmitter acetylcholine. The activity can easily be quantified [17] using the substrate analogue acetylthiocholine, since the product of the reaction, thiocholine, can be revealed with dithiobisnitrobenzoic acid, producing a yellow color by reaction with thiol groups.

We recently reported first qualitative attempts to control the activity of encapsulated β -lactamase via reconstitution of OmpF into the shell of the liposomes [1,11,19]. The different systems investigated (liposomes, polymer-stabilized liposomes and block copolymer vesicles) all showed the same activity within the experimental error [1]. A more precise quantification can be achieved with encapsulated acetylcholinesterase, and a summary is given in Table 1. For the lipid vesicles in absence of the porin, the substrate was able to diffuse slowly across the membrane, resulting in a small activity of AChE. Free AChE had an order of magnitude higher activity. Reconstitution of about 10 trimeric channels per liposome allowed the substrate to penetrate. However, at the rather low substrate concentrations investigated, the turnover of the enzyme is faster than permeation through the channel, so that acetylcholinesterase showed a reduced activity.

6. Discussion

Here, we demonstrated that liposomes can be used as nanometer sized bioreactors as schematically shown in Fig. 1. In these liposomes, we encapsulated different enzymes—among them β -lactamase and acetylcholinesterase—and showed the functionality of the enzymes. In combination with channel forming proteins, in our case porins, we can control the permeation of the substrate through the wall and thus adjust the rate of the reaction in the interior. The inherent instability of conventional liposomes could be overcome by polymerization of hydrophobic monomers inside their lipid bilayers. This leads to a quasi-two-dimensional polymer network structure, on which the lipids can freely glide. Another approach is to formulate block copolymer vesicles, which later can be cross-linked via polymerizable groups at the water-soluble blocks [19]. Very promising for future technological application is the fact that the membrane proteins and the encapsulated enzymes remain fully active despite the artificial surrounding and survive even the crosslinking procedure.

Currently, we are working on improving the selectivity of the capsule wall. The availability of structural information for several porins to a few Å resolution [3] facilitates enormously the interpretation of measurements. Furthermore, it permits the design of more efficient or more specific mutants by molecular engineering in the search for the best performer. For example, it is possible to create affinity sites necessary for specific sensors. Recently, the passage of isolated single stranded DNA through alpha-toxin channels was reported [22]. This stimulated the attempts to develop this observation towards a sequencing technique [23]. Although the realisation might be far in the future, the use of a channel as a sensor itself is highly interesting. Currently, we investigate the controlled opening and closing of specific porins using bacterial phages recognizing a site on the porin [1].

These results indicate that the nanoreactors represent a new type of delivery devices for applications in pharmacology and diagnostics. For applications of this kind, it is especially important

that such systems provide a constant release of substances over an extended period of time.

It has to be emphasised that the systems introduced in this study are representative examples of a novel brand of nanometer-sized bioreactors. In fact, nature provides many more specific, unspecific or ligand gated channels, which can be reconstituted in the same way, providing a unique tool to control permeation across the nanoreactor shells. We believe that the principle of using the protective feature of such nanocontainers in combination with controlled permeability either by natural or genetically modified channels or pumps will have many future applications. For example, the encapsulation could be extended to antibodies and thus decrease the immunogenicity of the enzyme when injected in plasma. We hope that this will allow therapeutic use of enzymes obtained from a much wider range of sources.

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